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2. Patent application number *(The Patent Office will fill in this part)* — 9826701.6 — 05 DEC 1998

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — UNIVERSITY OF NEWCASTLE, 6 Kensington Terrace, Jesmond, NEWCASTLE UPON TYNE

   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — 6594709001

4. Title of the invention — MICROCELLULAR POLYMERS AS CELL GROWTH MEDIA AND NOVEL POLYMERS

5. Name of your agent *(if you have one)* — MARKGRAAF PATENTS LIMITED

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — The Crescent, 54 Blossom Street, YORK, YO24 1AP

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MARKGRAAF PATENTS LIMITED

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MICROCELLULAR POLYMERS AS CELL GROWTH MEDIA AND NOVEL POLYMERS

The present invention relates to microcellular polymers as cell growth media, biologically active systems comprising the polymers and biological cells, a method for cell growth in the materials, the use thereof as implants in the human or animal body or for *in vitro* studies, novel processes for the preparation and modification of microcellular polymers and the polymers obtained thereby.

More specifically the present invention relates to microcellular polymers having porosity in excess of 75%, in the form of a pore and pore interconnect structure having relative pore and interconnect dimensions d/D as cell growth media for three dimensional cell growth throughout the polymer, the system comprising this, method for growth and use thereof, and novel processes for the preparation thereof and polymers obtained thereby.

Microcellular synthetic organic/inorganic/natural materials in which the pores are interconnected are often used as a support to grow plant or animal cells or enzymes.

Tailoring of a microcellular support for a specific cell growth application is often difficult with naturally forming microcellular materials. Recently efforts have been made to grow cells on newly developed microcellular materials, which are prepared through a high internal phase emulsion (HIPE) polymerisation route in which the phase volume of the dispersed phase is greater than about 75% and subsequently polymerising and/or cross linking

to obtain a rigid structure, offering many advantages. These materials have been described both in open literature and in patent disclosures, and they are referred to as polyhipe polymers (PHPs).

It is stated that the important features of these materials are:

1. The pore volume can be as high as 97% (for practical purposes);
2. Pores are interconnected;
3. Pore and interconnect sizes can be controlled accurately and independently;
4. Additional polycondensation reactions can be achieved;
5. Crosslinking can be achieved with proteins, polymers, silicates or organic polymers plus a wide range of porous materials can be obtained;
6. Post polymerisation/crosslinking modification of PHP is possible which is further facilitated by using functional monomers at the emulsification stage;
7. PHP can be obtained in block and/or in particulate form;
8. Since HIPEs can be pumped, it is possible to form moulded structures.

US patent 5,071,747 describes the preparation of microcellular polymeric support materials with average void (pore) diameter within a range of from 1 to 150 μm interconnected by holes (interconnects). Hole size (d) is related to the void size (D) and their ratio can be controlled in the range of $0 < d/D < 0.3$. The control of d/D ratio is through the control of the surfactant concentration and by the addition of electrolyte, mainly $CaCl_2$ in the range of 10^{-4} molar to 5 molar. The electrolytes are selected from soluble halides

and sulphates. The function of the electrolyte is claimed to be the control of the size of the holes and to improve stability of the emulsion.

In stage-1, the oil and aqueous phases are introduced under deformation (agitation) and in stage-2, the resulting HIPE is homogenised under deformation (agitation). Although the rate of introduction of the phases (i.e. dosing time (t_D)) of the dispersed phase into the batch mixer, and the subsequent homogenisation time (t_H) of HIPE is quoted in US patent 5,071,747, the mixing conditions are not specified. Polymerisation is followed by introduction of cells.

US 5,071,747 discloses isotropic (non-directional) plant and animal cell growth in three dimensions (3D) in polyvinyl Polyhipe Polymer (PHP), the generic name for the micro porous material. Reactants or nutrients are provided via inter connecting holes (interconnects) within the PHP to access voids (pores) in which cells are grown or reacted to produce products. The PHP is used purely as a cell growth medium allowing normal cell functioning within voids, using micro capillaries formed by holes as access channels. Voids and holes were provided having void diameter of 6-12 times the diameter of cells to be introduced and grown and hole diameter of 3-6 times, i.e. void diameter 45 micron and hole diameter 15 micron in the case of growth of yeast cells of the order 5 micron. In these cases, however cell growth was predominantly surface growth. In a further example PHP having porosity of 90%, void and hole diameter of 30 micron and 10 micron respectively was used for fungal growth which was found to penetrate the porous polymer network.

Nevertheless the literature gives only limited teaching with regard to controlling pore and interconnect size. In US 5,071,747 above it is not stated how the claimed dimensions were achieved and in fact it would not be possible to obtain pore dimensions in excess of 50 microns using the limited information given, nor would it be possible to control or pre-determine a particular pore size within the range 1-50 microns without extensive experimentation.

The usefulness of PHP materials for cell growth remains therefore extremely limited and there is a need for materials and methods for preparing more sophisticated polymers enabling more sophisticated cell growth.

We have now surprisingly found that coherent cell growth may be achieved within PHP polymers to provide a co-operating multi cell system which is adapted for a number of uses and that novel PHPs may be obtained having novel properties adapted for biological and non-biological uses.

Accordingly in the broadest aspect of the invention there is provided micro cellular polyhipe natural or synthetic polymer in the form of a homogeneous cross linked open cellular material having porosity greater than 75%, comprising pores of diameter 1 to 10,000 micron and pore interconnects wherein the polyhipe is a scaffold for multicell growth in three dimensions wherein the polymer pores and interconnects are comprised in a plurality of distinct or interpenetrating zones which are adapted to regulate cell positioning and morphology whereby cell growth is constrained within and/or extends throughout plural zones in directional and/or nondirectional manner to provide a multiple cell structure for biomedical applications.

Preferably zones are adapted for growth of multiple cell types independently constrained within and/or extending throughout multiple zones respectively.

The important parameters that promote cell growth are the pore and interconnect sizes and chemical and physical characteristics of the support surface. In some cases, the cell growth may be anisotropic (directional) and therefore the pores of the cell support may be required to be in the form of micro-channels with interconnects to provide cell-communication. The walls of the micro-channels can be (bio)degradable so that subsequent cell fusion can be obtained after (bio)degradation.

Biomedical applications include any application in which the materials interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body. The materials may therefore be envisaged for a range of applications for example the manufacture of contact lenses, dental fillings, cochlea implants, vascular supports including heart valves and cardiac pace makers and drug delivery skin patches and the like.

Reference herein to a scaffold is to a porous material which provides support and allows a load bearing function on the scaffold at an early stage as the scaffold takes the load until cells are grown within a scaffold and have developed extracellular matrix to gain load bearing ability and would stand loads and cells.

Cell growth may be of cells which are introduced into the scaffold or migrate into the scaffold, for example in the case that a scaffold is used for synthetic growth or is used for natural cell growth by migration of cells from surrounding tissue.

Reference herein to polyhipe is to any natural or synthetic polymer comprising pores and interconnects as hereinbefore defined obtained by polymerising a high internal phase emulsion.

Reference herein to pores and interconnects in the polymer is to empty pores or cells with pore interconnects therebetween, which may be empty or may contain dissolved or dispersed materials. Interconnects may form a micro capillary system. Pores and interconnects are distinguished by their relative magnitude and dimensions as herein below defined. The total porosity of the material is the combined empty space provided by the sum of all pores and interconnects.

Reference herein to zones within the polymer is to distinct or interpenetrating regions characterised by the form, location, magnitude or other property of pores and interconnects comprised in the zone. For example one or more zones are provided at the polymer surface, within its bulk matrix, at the interface between polymer and internal phase, between adjacent pores and/or interconnects of different form or dimension or adapted for growth of different cell types. Zones are distinguished by boundaries which may be between or contained within adjacent pores and/or interconnects in respective zones.

Preferably polymer according to the invention is suited for growth of multiple cell types to provide a multi zone cell structure in which selected cell types are confined to the specific boundary whilst other cell types grow throughout the structure across boundaries presented by the scaffold. By this means the polyhipe scaffold provides for a multiple cell system extending

throughout the scaffold, optionally including intrainterconnect or intramicrocapillary growth and exhibiting zoning and optionally interzoning of biological cells.

The polymer of the invention is moreover able to encourage natural zoning of cells by migration within, throughout and between zones which have been tailored for preferential migration of desired cell types.

The polymer of the present invention may be obtained from any desired natural or synthetic monomers, oligomers and mixtures thereof which exhibit bio-compatability. Polyhipes are commercially available or may be prepared using methods as disclosed in US 5071747 and in additional patent publications referred therein or as hereinbelow described.

The generic polyhipe polymer which is commercially available comprises polyvinyl polyhipe and is made up of oil phase monomers styrene, divinyl benzene (DVB) and surfactant (Span 80 sorbitan monooleate), and may be in rigid or flexible form depending on the relative proportions of monomers, additionally in flexible form including monomer 2-ethylhexyl acrylate, and in the aqueous phase an amount of potassium persulphate as aqueous phase initiator.

The polymer of the invention may be natural or synthetic, soluble or insoluble, optionally (bio)degradable crosslinked polymer, preferably selected from proteins and cellulose, polyacrylamide, polyvinyl in rigid or flexible form, poly(lactic acid), poly(glycolic acid), polycaprolactone and polyacrylimide.

Commercially available polyhipe typically has pore diameter in the range 1-50 micron.

Polyhipe according to the invention may have pore diameter in the range 0.5 or 1-10,000 micron. A preferred lower range limit is 30 micron, more preferably 50, for example 150 micron. A preferred upper range is 1000 micron, more preferably 500 micron. Polyhipe of particular pore diameter may be obtained by methods described hereinbelow, and may have any desired ratio of interconnect to pore diameter, for example in the range $0 < d/D < 0.4$, preferably in the range $0.1 < d/D < 0.4$. Interconnects may have diameter in a range of 10-1000 micron, preferably 10-200 micron. Extensive elongate micro capillary interconnects of diameter as low as 10 micron may be provided, the capillary interconnects being separated by the microcellular polymer. A zone comprising the interface between a capillary wall and the bulk polymer may provide a surface layer of any desired thickness for growth of cells of any desired size. An interface having a thin surface layer of the order of 0.5 micron is particularly suited for growth of neurons (nerve cells) or muscle cells in which directionality is important. The interface having smaller pore size than the bulk provides an ideal zone for growth of cells forming a lining, for example cells lining the blood vessels or for growing endothelial cells on the interface surface.

Preferably pore size and inter connect size are selected according to the type of cell to be grown and the type of growth, i.e. with or without penetration, confined to a boundary or crossing boundaries between zones. Pore size in zones intended for growth of cells throughout the zone is preferably of diameter 2 – 3 times the diameter of cells to be grown, for example 2.5 times cell diameter. Without being limited to this theory, it is thought that this

ratio of dimensions is optimum for cell types, including cartilage cell types, whereby cells are able to grow and prosper, as indicated by production of collagen. For example cartilage cells of diameter 10 micron were grown in pores of diameter in the range 17-30 micron, specifically of 25 micron.

The polyhipe scaffold may be adapted to provide a desired surface characterised by means of the surface coating, using coating materials introduced *in situ* during polymerisation or post polymerisation. Any known materials typically used for coating polymers for use in cell growth may be employed, for example cell growth promoters such as hydroxylapatite or tricalcium phosphate, which also promotes biocompatability, other minerals, silica, collagen, hyaluran, polyethylene oxide, carboxymethyl cellulose (CMC), proteins, organic polymers, particles and the like. Coating *in situ* provides homogeneous coating throughout the scaffold. Post polymerisation coating, for example using laser ablation results in coating confined to the surface of the bulk matrix and may be particle coating.

The polyhipe scaffold may be constructed of any desired materials as hereinbefore defined. It has been found that cyclic mechanical straining of the support or the application of an electric field may also enhance the rate of cell growth. In most cases, the biodegradability of the support does not appear to be important and therefore the support material with growing cells can be implanted in the animal body without fear of rejection. However, when cell-cell fusion is required, (bio)degradability becomes important.

Therefore the polymer is preferably made of resiliently deformable or elastic materials or is rendered resiliently deformable or elastic by suitable means. Preferred materials are therefore thermoplastics which may be deformed in

suitable manner to influence cell growth. Preferably a polyhipe support as hereinbefore defined is suited for repeated stress and relaxation by means of oscillatory straining of the scaffold during cell growth. This has been found to have beneficial effects in cell growth rate promotion.

The polyhipe scaffold as hereinbefore defined may be electrically conductive or may be rendered electrically conductive by known means whereby it is adapted to conduct an electric current during cell growth. This technique is particularly advantageous for distinguishing certain cell types and promoting growth and fusion of particular cell types such as neurons and muscle cells.

The polyhipe scaffold may be biodegradable or may be rendered biodegradable by known means whereby it is adapted to be degraded by contact with any desired agent or by introduction into any desired environment, for example a specific biological agent or environment. Preferably the polyhipe scaffold is adapted for degradation by the hydrolysis or enzyme-catalysed hydrolysis of the polymer chains and cross link or by the erosion of the polymer. By this means the polyhipe scaffolds may be used as a scaffold to support a biologically active system for the duration of time required for that system to establish itself and be self supporting and thereafter degrade with no detrimental effects. Preferably a degradeable scaffold is used for growth of cell types including for example muscle cells. By this means a scaffold is adapted to position multiple cell types in pre determined zones and thereafter to degrade allowing self fusion to take place in the absence of the intervening scaffold whereby functioning biologicially active micro systems are created.

In a further aspect of the invention there is provided a polyhipe scaffold as hereinbefore defined comprising multiple cells characterised by growth in three dimensions as hereinbefore defined in polymer zones as hereinbefore defined.

The scaffold including cells may comprise any of the properties as hereinbefore defined.

Multiple cells may be any desired cell type selected from human, animal and plant cells. Preferably cells are human or animal cells and are representative of multiple cell types present in any organ, system or part of the human or animal body. Cells are preferably selected from isotropic tissue and bone cells present in cartilage, cornea, marrow and the like, anisotropic cells such as nerve, muscle, blood vessel cells and the like. Cell type includes for example fibroblasts, chondrocytes, osteoblasts, bone marrow cells, hepatocytes, cardiomycytes, neurons, myoblasts, macrophages and microvascular endothelium cells. Chondrocytes grow for example throughout surface and matrix zones in the polyhipe, and osteoblast growth is confined to the surface.

In a further aspect of the invention there is provided a biologically active system comprising a polyhipe scaffold and multiple cells as hereinbefore defined adapted to provide normal cell functioning associated with a natural biologically active system present in the human or animal body. A biologically active system may comprise the polyhipe scaffold intact or in partially degraded state as hereinbefore defined adapted for fusion or assimilation into an environment.

In a further aspect of the invention there is provided a method for growth of multiple cells in a polyhipe scaffold as hereinbefore defined comprising providing cells on or in the scaffold in a controlled environment and providing a suitable nutrient adapted for growth in known manner. The method of the invention may include any technique for growth promotion, positional control and the like adapted to provide a structural system as hereinbefore defined.

In a further aspect of the invention there is provided a polyhipe scaffold adapted for multiple cell growth as hereinbefore defined, comprising multiple cells as hereinbefore defined or a biologically active system as hereinbefore defined for use as an implant *in vivo* in the human or animal body or as modules for *in vitro* studies mimicking a part of the human or animal body or for use in a growth environment for further systems as hereinbefore defined, for example for the growth of organ cells in the cell side of the module in order to simulate organs.

According to this aspect of the invention *in vitro* organs may be produced using the micro capillary interconnects of the scaffold as hereinbefore defined for nutrient circulation and waste product removal while growing cells in the bulk pores and additionally or subsequently when anisotropic cell growth is needed for example to mimic cardiovascular systems and the like, employing circulation of nutrients and waste products removable through the bulk pores of the micro porous scaffold while growing cells in the micro capillary interconnects. It is a particular advantage of the invention that this allows growth and sustains cell functioning for prolonged periods. The use of the scaffolds and systems as hereinbefore defined as *in vitro* organs may be harnessed for testing of pharmaceuticals and any substances to be

introduced into the human or animal body, for genetic engineering, for studying mechanisms associated with disease and the like under controlled conditions and the like. This is particularly advantageous in eliminating the need for animal testing. *In vitro* modules as hereinbefore defined for specific types of multiple cells and applications may be provided and may be associated with instrumentation to provide the necessary physiological conditions.

It should be appreciated that the invention derives from the finding that polyhipe scaffolds as hereinbefore defined may be provided with controlled pore dimensions and diameters in zones as hereinbefore defined, such that for example one cell type penetrates the scaffold and a second cell type may grow on the surface. This enables controlled cell growth and positioning in manner to create a multiple cell structure which is adapted to co-operate to provide biological activity.

In a further aspect of the invention there is provided a process for the preparation of microcellular polyhipe natural or synthetic polymers as hereinbefore defined comprising in a first stage the formation of a high internal phase emulsion (HIPE) of dispersed phase in continuous phase, wherein the dispersed phase may be void or may contain dissolved or dispersed materials, and monomers, oligomers and/or pre-polymers are present in the continuous phase, homogenisation and polymerisation thereof, by means of in the first stage introducing the dispersed phase by controlled dosing into the continuous phase with controlled mixing at controlled temperature to achieve an emulsion, and subsequently homogenising for controlled period under controlled deformation and polymerising, under controlled temperature.

The process of the invention enables a vast range of pore sizes to be obtained by controlling processing technique and compositional conditions. The pore sizes are obtained in roughly 3 groups: they are; small pore size: 1-10 μm; large pore size: 11-200 μm and very large pore size: 201-10,000 μm.

Very small pore size emulsions (approaching 1 μm) are obtained using very high deformation rate flows in which the flow is predominantly extensional and the emulsification temperature is as low as possible. Large pore size emulsions (approaching 200 μm) are obtained at high temperatures and just above the critical deformation rate below which the emulsion will fully or partially invert, for example to an oil-in water type system. The critical deformation rate may be determined by varying, for example the rate of addition or mixing for a given system.

These emulsions should also be processed in a short time using predominantly shear flows. Very large pore emulsions (approaching 10,000 μm) are obtained through the method of controlled pore coalescence during polymerisation in which the aqueous (dispersed) phase contains a known amount of water soluble polymer. In this case, the pore size is dictated by the size of the pores before the start of coalescence, temperature of polymerisation and concentration, molecular weight and type of water soluble polymer.

The emulsion may be obtained from any desired immiscible phases forming a continuous and a dispersed phase, preferably from aqueous and non-aqueous phases, more preferably aqueous and oil phases. The emulsion obtained may be an aqueous in oil emulsion or oil in aqueous emulsion.

The process according to the invention may be used for the preparation of any desired polymers as hereinbefore defined.

According to this aspect of the present invention, it has surprisingly been found that by means of controlled dosing the dispersed phase into the continuous phase, it is possible to achieve the desired emulsion. In a batch mixer, dosing of the dispersed phase is preferentially conducted from the bottom of the mixer, using either single or multiple entry points. Multiple entry feed resulted in larger pore emulsions. If the dosing rate was very fast, mixing created by the emerging jet of aqueous phase was too severe and therefore the emulsion pore size decreases. Therefore, this combination of multiple-feed points with a relatively prolonged dosing created large pore emulsion. After the completion of dosing, the emulsion should be homogenised but if the homogenisation period was long, pore size decreased.

Controlled mixing as hereinbefore defined may be critical or extended. Critical mixing is sufficient mixing to cause transition from oil in aqueous to aqueous in oil emulsion. Critical mixing is obtained by use of a homogeneous mixing field whereby pore size is substantially uniform preventing emulsion breakdown and phase inversion.

Mixing may be by any means suited to provide a homogenous mixing field substantially throughout the total volume of the two phases and is preferably by multiple blade, multiple jet and the like mixing.

It has surprisingly been found therefore that, contrary to the teaching of US 5,071,747, the achievement of a stable emulsion with large pore diameter is obtained by minimising the intensity of mixing. According to the invention it has been found that by means of dosing, homogeneous mixing and the like a stable emulsion may be obtained dispensing with the need for intense mixing.

Dosing, emulsifying and homogenisation may be conducted at any suitable temperature and preferably at elevated temperature below the boiling point of the lowest boiling phase. An aqueous phase as the lowest boiling phase, boils at around 100°C. It has been found that the process of the invention employing emulsification temperature of 60°C or greater results in polymers being obtained having pore size in excess of 60 micron. Increase of emulsification temperature above 60°C results in dramatic increase in pore size by an amount greater than that achieved for a similar increase in temperature below 60°C.

The maximum emulsification temperature may be greater than the normal boiling temperature of the lowest boiling phase, for example the lowest boiling phase may include any suitable component adapted to raise the boiling point. Preferably the aqueous phase includes an electrolyte which is stable at 100°C and is potentially inert. By this means the process is preferably carried out with use of homogenisation temperature in the range 60-150°C, more preferably 80-140°C, most preferably 90-140°C.

The process may be carried out with use of additional aqueous or oil phase initiators, cross linking agents, fillers and the like and it is preferred that these are stable at the maximum operating temperature as hereinbefore

defined. Selection of initiators, cross linking agents and the like is made with reference to the acceptable viscosity of the phases for emulsifying and homogenising. It may be acceptable to reduce the amount of cross linking agent required by use of a proportion of pre polymers and partly cross linked pre polymers, optionally with the use of a suitable oil phase filler to increase oil phase volume and reduce effective viscosity.

Preferably the process of the invention is characterised by use of an initiator in the oil phase, together with or in place of an aqueous phase initiator as known in the art.

Preferably the process is carried out with use of oil phase fillers and allowing operation at high emulsification temperature and with use of minimum cross linking agent. This has the further advantage that oil phase filler such as high boiling point hydrocarbon may be leached out after polymerisation increasing the interconnecting micro capillary size.

Electrolytes may be for example calcium chloride or minerals such as hydroxy apatite.

Aqueous phase initiators may be employed for operation at lower temperatures and include sodium or potassium persulphate.

For operation at elevated temperature above 80°C oil phase initiators are preferably used for example, 1,1-azobis (cyclohexanecarbonitrile).

Cross linking agent may be for example divinylbenzene (DVB). If the polyhipe is required to be biodegradable, hydrolyzable crosslinks can be

obtained. These crosslinking agents are ethylene diacrylate, N-N'-diallyl tartardiamide, N-N (1,2, dihydroxyethane) - bis -acrylamide, and N-N'-N" - triallyl citrictriamide. However, in this case of biodegradable crosslinkers, the polymer itself should be biodegradable. These polymers are poly (lactic acid), poly (glycolic acid), poly ϵ-Capri lactone and polyacrylimide.

When water soluble polymers are needed to form the microcellular structure, they need to be crosslinked . In this case of such polymers, monomer (such as acrylamide) is dissolved in water and a HIPE emulsion is formed dosing this monomer solution into a hydrocarbon liquid such as hexane or toluene in the presence of suitable surfactant, initiator and crosslinker.

Proteins and cellulose can also form microcellular structures. In this case, these materials together with a suitable emulsifier are dissolved in a suitable aqueous phase (water for proteins and Schweitzer's reagent, $Cu(NH_3)_4(OH)_2$ for cellulose) and dosed into a hydrocarbon liquid to form a water continuous HIPE. Crosslinking is achieved by immersing the HIPE into a solution of glutaraldehyde (for proteins) or acid solution (cellulose).

Oil phase filler may be any high boiling point hydrocarbon.

The process may include introduction of any suitable modifier as hereinbefore defined prior to or subsequent to polymerisation. For example minerals such as hydroxy apatite may be introduced in the dispersed aqueous phase and dosed into the continuous phase as hereinbefore defined. Alternatively a post polymerisation modification stage is employed, which may simply take the form of removing surfactant, electrolyte and unreacted monomer, coating, further polymerisation or reaction on the existing

polymer surface. Modifying agents and modification techniques are shown in the art.

Polymerisation is carried out under known conditions of time and temperature for the respective monomer, oligomer and or prepolymer to be polymerised, as known in the art.

In a further aspect of the invention there is provided a microcellular polyhipe natural or synthetic polymer in the form of a homogeneous cross linked open cellular material having porosity in excess of 75% comprising pores and pore interconnects formed by polymerising a high internal phase emulsion (HIPE) as herein before defined wherein average pore diameter is in excess of 50 micron. Preferably average pore diameter is in excess of 100 micron, more preferably in excess of 150 micro and is selected to be suitable for the desired polymer application. Pore diameters up to 10,000 micron are envisaged. Interconnect diameters are as hereinbefore defined.

In a further aspect of the invention there is provided polyhipe polymer as hereinbefore defined having average pore size as hereinbefore defined in the range 1-10,000 micron, wherein the polymer is modified during polymerisation thereof or post polymerisation to be electrically conducting, degradable, or comprises mineral distributed throughout the matrix or as a surface coating.

In a further aspect of the invention there is provided an apparatus for the preparation of a polymer according to the invention comprising a mixing vessel adapted to contain the continuous phase having multiple inlets for the introduction by dosing of the dispersed phase as hereinbefore defined, and

comprising means for homogeneous mixing as hereinbefore defined and comprising temperature elevating and regulating means.

The invention is now illustrated in non limiting manner with reference to the following examples.

Example A - preparation of polyhipe, using apparatus according to the invention.

The preparation of the emulsion was carried out in a batch mixer from an oil phase and an aqueous phase, dosed at a predetermined rate while the emulsion was stirred at constant rotational speed. Dosing rate, deformation rate, mixing rate were predetermined as a function of volume of respective phases, diameter of the batch mixer and of the impellers, rotational speed of the impellers and homogenisation time.

In order to eliminate the differences in performance of different mixing conditions we characterise the mixing through:

Dosing rate $R_D = \dfrac{V_A}{t_D}$

Deformation rate during mixing $R_E = \dfrac{V_A}{t_D\, V_o}$

Mixing rate $R_M = \dfrac{D1}{Do}\ \Omega$

Where:

V_A = Volume of aqueous phase added over a period of time t_D

V_o = Volume of the oil phase placed in the batch mixer

D_I = Diameter of the impellers

D_o = Diameter of the batch mixer

Ω = Rotational speed

We also define t_H as the homogenisation time and t_T as the total mixing time.

$t_T = t_D + t_H$

After the preparation of the emulsions under a given set of conditions as described in each of the following examples, the emulsion was allowed to polymerise at 60°C for 8 hours. Samples were further modified as described in the following examples.

The pore and interconnect sizes were determined using scanning electron microscope, using thoroughly dried and washed samples.

Example A1 - the effect of operating condition on the pore and interconnect size.

The oil phase contained 78% styrene, 8% DVB monomer and cross-linking agent and 14% Span 80 surfactant sorbitant monooleate while the aqueous phase contained 1% potassium per sulphate. Two flat paddle impellers (8 cm in diameter and 1.4 cm in width) were used in a mixing tank of 8.5 cm diameter. Impeller separation was 1 cm. 25 ml of oil phase is placed at the bottom of the tank and 225 ml of aqueous phase was dosed using 16 feed points. Temperature of the aqueous phase was ranged from -1.0 to 80°C.

Table 1 - The effect of operating conditions on the pore and interconnect size.

Temp (degC)	Pore Size(D) (μm)	Interconnect Size(d) (μm)	Dosing time(s)	Homogenisation time(s)	d/D
5	34	9	40	60	0.26
25	37	14	40	60	0.38
60	65	22	40	60	0.33
80	141	37	40	60	0.26
25	102	25	40	20	0.25
25	72	21	30	20	0.29
25	67	22	25	20	0.33
25	148	43	60	20	0.29

Example A2 – Effect of water soluble polymers on pore and interconnect size in controlled pore coalescence.

Emulsions were prepared using 78% styrene, 8% DVB and 14% Span 80 as the oil phase. The aqueous phase contained 1% potassium per sulphate and varying amounts of water soluble polymers, sodium carboxy methyl cellulose (CMC) or polyethylene oxide (PEO). Emulsification is at 25°C, water phase volume is 85% and the aqueous phase is delivered through a single inlet. The processing conditions were: $t_D = t_H = 6$ seconds, $R_D = 0.70$ ml/s, $R_E = 0.0067\ s^{-1}$ and $R_M = 4.7\ s^{-1}$.

Table 2 - The effect of water soluble polymers on pore size in controlled pore coalescence

Relative Molecular Mass	Water Soluble Polymer Concentration wt% of aqueous phase	Pore Size (μm)
Control	0	18
Sodium carboxylmethyl cellulose (CMC)		
90,000	0.5	22
90,000	1.0	260
250,000	1.0	1200
Polyethylene oxide (PEO)		
200,000	1.0	420
400,000	1.0	4300

Example A 3 - In-situ surface coating with hydroxylapatite

Emulsions were prepared using: (A1) 78% styrene, 8% DVB and 14% Span 80 or (A2): 15% styrene, 60% 2-ethylhexyl acrylate, 10% DVB and 15% Span 8. The aqueous phase contained either; (B1): 1% potassium per sulphate or (B2): 1% potassium per sulphate 0.5% hydroxylapatile (HA) and 15% phosphoric acid which is used to dissolve the hydroxylapatite (HA). Emulsification is carried out at 25°C, with water phase volume at 85% and the aqueous phase is delivered through a single inlet. The processing conditions were $t_D = t_H = 600$ seconds, $R_D = 0.83$ ml/s, $R_M = 4.7s^{-1}$ and $R_E = 0.0067\ s^{-1}$. After the emulsification and polymerisation, samples containing hydroxylapatite were soaked in 1M NaOH to preciptate hydroxylapatite. After precipitation, the samples were cleaned in water to retain pH = 7 and IPA and examined for their structure.

Table 3 - The effect of hydroxylapatite on pore and interconnect size.

Description	Oil and Aqueous Phase Compositions			
	A_1 B_1 (Rigid PHP)	A_1: B_2 (Rigid/HA PHP)	A_2: B_1 (Elastic PHP)	A_2; B_2 (Elastic/HA PHP)
Pore size D (μm)	22	38	9	17
Interconnect size d (μm)	2	11	1.5	6
d/D	0.09	0.29	0.17	0.35

Samples with compositions (A_1; B_1) and (A_1; B_2) are examined under SEM and their surface elemental analysis indicated uniform distribution of hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) as shown in Table 4.

Oil phase containing just styrene is referred to as rigid PHP and when oil phase contains 2-ethylhexyl acrylate, the resulting composition is referred to as elastic PHP.

Table 4 - Elemental analysis of the fracture surface of two polyhipe polymers with compositions (A_1; B_1) and (A_1; B_2)

Component	Unmodified PHP (A_1:B_1) - wt%	Hydroxylapatite coated PHP (A_1; B_2) wt%
Carbon	82	74
Oxygen	12	21.4
Calcium	-	3.2
Phosphorus	-	1.4

This in-situ hydroxylapatite coating of polyhipe is preferred since post-polymerisation soaking of the material in hydroxylapatite solution and subsequent precipitation appeared to form large but localised hydroxylapatite crystals within the polyhipe pores.

Example A 4 – Effect of oil phase composition on pore and interconnect size

An emulsion was prepared as described above, including 0.5% of oil phase initiator, 1,1'-azobis(cyclohexanecarbonitrile). Total volume and viscosity were substantially unchanged. The emulsion was processed and polymerised as described above but using conventional mixing times and rates as known in the art (Dosing rate R_D = 0.71ml/s, mixing rate, $R_M = 4.7\ s^{-1}$, mixing time $t_D = t_H$ = 600s), and the material properties evaluated. The results are shown in Table 5.

Table 5

Sample	Pore Size,D (μm)	Hole Size, d (μm)	d/D
Control polyhipe	15.8	2.3	0.15
Polyhipe with initiator	22.7	4	0.18

Example A 5 - Effect of oil phase soluble initiator on hydroxyl modified polyhipe.

An emulsion was prepared using 0.5 wt% hydroxylapatite solution (pH = 2.5 adjusted with phosphoric acid) without any aqueous phase initiator. The oil phase contained 77.5% styrene, 8% DVB, 14% Span 80 and 0.5% azobisisobutyronitrile (AIBN) as oil phase initiator.

All other experimental conditions were the same as Example 3. In Table 6 the hydroxylapatite modified polyhipe is compared with the corresponding material in Example 3 (i.e., rigid/HA PHP).

Table 6

Sample	Pore Size D (μm)	Interconnect Size d (μm)	d/D Ratio
Rigid/HA	38	11	0.29
Rigid/HA AIBN initiated	30	12	0.40

The above table indicates that the presence of oil phase initiator does not substantially affect the pore and interconnect sizes. However, the elemental analysis of the polyhipe surfaces using EDAX (energy dispersive analysis with X-rays) show that the presence of HA is more pronounced if oil phase initiator is used. These results are shown in Table 7.

Table 7

Component	Overall analysis on HA modified polyhipe with AIBN	Overall analysis on HA modified polyhipe
Carbon	63.09	74
Oxygen	22.49	21
Phosphorus	2.98	2
Calcium	9.70	3

The concentration of calcium ions is greater than for the control which did not contain AIBN

Example B 6 – multiple cell growth in polyhipe

Chondrocytes (obtained from bovine metacarphalangeal joint) human osteoblasts cells (HOB) and 3T3 fibroblast rat cells were obtained. Two samples of polyhipe, without and with HA were cut into discs, washed and thoroughly sterilised. Thermanox discs were used as control. The discs were soaked in the respective cell culture medium for 24 hours and placed in well plates. 3T3, HOB and chondrocytes cells were each seeded onto the discs at a densities of 100,000, 100,000 and 500,000 cells per ml respectively. The plates were then incubated at 37C in 5% CO2 for up to three weeks. Samples were examined after 1,7,14 and 21 days by SEM and for histology.

Results

The 3T3 which grew on the rigid polyhipe can be seen to have multiplied rapidly, by day 1 a confluent layer was present on both types of polyhipe. This layer remained throughout the duration of the experiment. From histology it could be seen that there was no penetration, in some samples by day 7 and in all samples by day 14 the cell layer had begun to leave the surface of the polyhipe.

The HOB cells showed well spread morphology on day 1 under SEM, the cells were bridging the interconnects on the surface of the polyhipe, opposed to growing into the interconnects. By day 7 all samples had a confluent layer on the surface. From histology was noted that there was slight penetration, only to the depth of one pore in the samples containing HA. The layer remained securely attached to the surface throughout the duration of the experiment.

The chondrocytes at time points of 1 and 7 days adhered to the polyhipe discs and showed rounded morphology on both types of polyhipe, compared to the Thermanox[R] control where the cells had spread out and become fibroblastic in appearance. By day 14 there was signs of flatter cells and by day 21 there were confluent layers on some samples. The cells on the HA polyhipe could be observed to flatten at early time points compared to the unmodified polyhipe. From histology it was noted that from day 5 onwards there was penetration in both polyhipes and cells were present within the 3D structure, however the HA polyhipe had greater penetration and visually seemed to contain more cells. The cells which had penetrated the unmodified polyhipe were fewer in number however were retaining their rounded morphology.

From the results of Safinin O staining it can be shown that healthy chondrocytes were present in both the centre and the periphery of the polymer matrix. There were viable cells secreting GAG both in the polymer and on the periphery.

The rate of chondrocyte growth studies (as obtained from DNA assays) indicate that the hydroxylapatite modified polyhipes yield significantly faster growth compared with unmodified polyhipes as shown in Table 8.

Table 8

Type of Assay	Cell Lysis (mg DNA/ml)	
Time (day)	PHP	HA-PHP
1	3.1	3.2
5	3.6	4.4
10	3.6	9.5

Example B7 – Effect of Pore Size on Cell Growth

Five types of styrene / 2-ethylhexyl acrylate Polyhipe Polymers were produced with average pore size of 8, 17, 31, 34 and 89 μm. These samples were then seeded with chondrocytes and the effect of pore size was noted. The results are shown in Table 8.

Table 8. Effect of PHP pore size on the cell penetration and collagen II production.

	Pore Size (μm)				
	8	17	31	45	89
Depth of Penetration (μm)	17	508	367	67	45
Relative Collagen II Production	23	48	42	12	9

The production of Type II collagen is an indication of the correct physiological cell function. This example indicates that, for chondrocytes which have cell size of approximately 10 μm, the optimum pore size is approximately 25 μm. If the pore size is too low, the cells can not penetrate the polymer and if the pore size is too large, the cell morphology changes from rounded to a flat and fibroblastic appearance. These fibroblastic cells proliferate rapidly and form a layer on the surface rather than penetrating the polymer.

Example 9B – Macrophage Activity on Sulphonated Polyhipe Polymer

The biocompatibility of the materials can be tested by exposing them to macrophages. The response of macrophages to a material is assessed by a) Morphological changes to macrophages, b) Hydrogen peroxide production by macrophages and c) Beta glucuronidase production by macrophages. Macrophages are spherical *in vitro* and any deviation from this response is a negative response. Excessive production of hydrogen peroxide and beta glucuronidase are also negative responses.

A styrene / 2-ethylhexyl acrylate Polyhipe Polymer is produced. Some of this material is sulphonated (degree of sulphonation is 12%) and subsequently neutralised using sodium hydroxide. Macrophages are seeded on these polymers and their morphological and hydrogen peroxide and beta glucuronidase production capacities are assessed after six hours. The results are shown in Table 9.

Table 9. Effect of substrate on macrophage activity

Substrate Type	Morphology	Relative Hydrogen Peroxide Production	Relative Beta Glucuronidase production
PHP	Round	370	47
Sulphonated PHP	Flat	975	684
TCP*	Round	295	93

- **TCP = Tissue Culture Plastic**

Table 9 indicates that sulphonated PHP yields very high level of negative activity and therefore such polymers may be used as negative standard in which the activity is reduced as the degree of sulphonation is decreased.

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